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Article

Enhanced Orexin Receptor-2 Signaling Prevents Diet-Induced Obesity and Improves Leptin Sensitivity

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SUMMARY

The hypothalamic orexin neuropeptide acutely promotes appetite, yet orexin deficiency in humans and mice is associated with obesity. Prolonged effects of increased orexin signaling upon energy homeostasis have not been fully characterized. Here, we examine the metabolic effects of orexin gain of function utilizing genetic and pharmacologic techniques in mice. Transgenic orexin overexpression confers resistance to high-fat diet-induced obesity and insulin insensitivity by promoting energy expenditure and reducing consumption. Genetic studies indicate that orexin receptor-2 (OX2R), rather than OX1R signaling, predominantly mediates this phenotype. Likewise, prolonged central administration of an OX2R-selective peptide agonist inhibits diet-induced obesity. While orexin overexpression enhances the anorectic-catabolic effects of central leptin administration, obese leptin-deficient mice are completely resistant to the metabolic effects of orexin overexpression or OX2R agonist infusion. We conclude that enhanced orexin-OX2R signaling confers resistance to diet-induced features of the metabolic syndrome through negative energy homeostasis and improved leptin sensitivity.

INTRODUCTION

Animals employ genetically and physiologically determined homeostatic mechanisms to prevent excess weight gain; however, this regulation may be circumvented by environmental and hedonic factors. The ready availability of palatable, calorically dense food and the reduced need for physical activity in modern life have resulted in a pandemic of obesity. Rodent models have elucidated the mechanisms and modulators of body weight homeostasis such as the fat tissue-derived satiety hormone leptin (Enriori et al., 2007; Myers et al., 2008). Leptin centrally regulates body weight by suppressing food intake and permitting energy expenditure. While absence of leptin in rodents causes hyperphagia, obesity, and diabetes, most human obesity is associated with hyperleptinemic leptin resistance.

Orexins (also known as hypocretins) are lateral hypothalamic neuropeptides that are upregulated with fasting and can acutely promote appetite when administered into the central nervous system (Sakurai et al., 1998). The two receptors for orexin, type 1 (OX1R) and type 2 (OX2R), show differential affinity for the products of the prepro-orexin gene, orexin-A and orexin-B (Sakurai et al., 1998). OX1R and OX2R exhibit distinct expression patterns, indicating distinct roles in behavior and metabolism. The arcuate nucleus of hypothalamus (ARH) is a point of convergence for both orexin and leptin signaling, which modulate the activities of neuropeptide regulators of food intake and metabolism such as neuropeptide Y (NPY), agouti-related peptide (AGRP), and pro-opiomelanocortin (POMC). Pathologic leptin resistance may be mediated by changes in second messengers, including the long form of leptin receptor (LEPR), downstream signal transducer and activator of transcription-3 (STAT3), or the feedback suppressor of cytokine signal-3 (SOCS3) (Horvath, 2005; Myers et al., 2008).

Central administration of orexin neuropeptides to rodents acutely promotes appetite, and prepro-orexin deficiency or postgestational ablation of orexin neurons in mice causes modest reductions in food intake. However, orexin-deficient mice also exhibit narcolepsy, inactivity, and obesity, indicating that orexin may exert an overall catabolic influence upon energy balance (Hara et al., 2001, 2005; Willie et al., 2001). Narcoleptic human individuals (the majority of which are orexin deficient) have also been reported to have greater body mass index and higher incidence of metabolic syndrome (Nishino, 2007). This effect of orexin upon energy balance may be primary, since orexin-deficient narcoleptic patients showed higher body mass index than otherwise clinically indistinguishable narcoleptics with normal orexin levels (Nishino et al., 2001).

As the conclusion that orexin promotes negative energy balance derives indirectly from loss-of-function studies, we utilized genetic and pharmacological methods to directly examine

whether increased orexin signaling promotes negative energy balance. First, using *CAG/orexin* transgenic mice (Mieda et al., 2004) that overproduce orexin neuropeptides from an ectopically expressed transgene, we examined the effects of constitutively increased orexin signaling upon diet-induced obesity by measuring adiposity, locomotor activity, and other metabolic parameters. To differentiate the role of each receptor pathway, we also examined the effects of the *CAG/orexin* transgene upon *OX1R* knockout mice (*OX1R*$^{-/-}$, Kisanuki et al., 2001) and *OX2R* knockout mice (*OX2R*$^{-/-}$, Willie et al., 2003). Results of genetic studies were then verified and extended pharmacologically using a selective agonist for OX2R. Finally, we sought to determine the effect of the *CAG/orexin* transgene upon leptin-deficient *ob/ob* mice, examined the sensitivity of *CAG/orexin* mice to central leptin administration, and investigated the effects of selective OX2R agonism upon *ob/ob* mice.

RESULTS

Expression of Orexin Peptide in *CAG/Orexin* Mouse

Previous results with *CAG/orexin* transgenic mice revealed multifold increases in both orexin-A and orexin-B peptides in whole brain extracts (Mieda et al., 2004). Immunohistochemical localization of orexin-A in the brain of *CAG/orexin* mice demonstrates ectopic peptide production in medial, basal, lateral, and suprachiasmatic hypothalamic nuclei, nucleus accumbens, globus pallidus, hippocampal formation, ventral tegmental area, and locus coerulus (Figures S1 and S2, Table S1). All of these locations have previously been implicated as participants in networks controlling various homeostatic, circadian, learned, and/or hedonistic aspects of food intake, taste preference, or energy homeostasis (Saper et al., 2002). Previous results demonstrated that *CAG/orexin* transgene insertion was sufficient to rescue the narcolepsy/cataplexy phenotype of mice lacking endogenous orexinergic neurons (Mieda et al., 2004). Thus, the *CAG/orexin* transgene produces functional peptides that can activate orexin receptors.

The physiological relevance of peripheral actions of orexins, if any, remains controversial (Heinonen et al., 2008). In spite of the use of a general promoter for orexin overexpression, we found that *CAG/orexin* mice exhibited ectopic orexin-A immunoreactivity in a limited set of peripheral tissues, including thyroid gland, adrenal cortex, and some pancreatic islets. No evidence of ectopic expression was encountered in other metabolic tissues such as brown and white adipose, liver, or skeletal muscle (Figure S3, Tables S2 and S3).

CAG/Orexin Mice are Resistant to Diet-Induced Obesity

To examine the effect of increased orexin on body weight, *CAG/orexin* transgenic mice and wild-type littermate mice were fed either a low- or a high-fat diet. In both male and female mice, the body weights of wild-type mice were significantly higher when fed a high-fat diet compared to a low-fat diet. However, mice overexpressing orexin did not show a significant difference in body weight growth between a low-fat diet and a high-fat diet (Figures 1A and 1B). Thus, wild-type mice are susceptible to diet-induced obesity, whereas *CAG/orexin* mice are quite resistant.

To determine which receptor pathway mediates the antiobesity effect of orexin overexpression, we crossed *CAG/orexin* transgenic mice to *OX1R*$^{-/-}$ and *OX2R*$^{-/-}$ lines. We compared the effects of isolated orexin-OX2R signaling (in *OX1R*$^{-/-}$ mice and *OX1R*$^{-/-}$; *CAG/orexin* mice) versus isolated orexin-OX1R signaling (in *OX2R*$^{-/-}$ mice and *OX2R*$^{-/-}$; *CAG/orexin* mice) upon growth curves. Figures 1C and 1D show that increased OX2R activation is sufficient to mediate the preponderance of resistance to diet-induced obesity. On the other hand, in both sexes, increased OX1R activation alone does not significantly protect from development of obesity (Figures 1E and 1F). Unlike differences in body weight, there were no significant differences in linear growth among the various genotypic groups (data not shown). Thus, OX2R signaling selectively mediates the antiobesity effect of orexin overexpression in mice challenged with a high-fat diet.

CAG/orexin-transgenic male mice were also resistant to aging-related adiposity, while wild-type male mice fed a low-fat diet showed continuous weight gain during aging (Figure 1A). In spite of similar growth curves before 18 weeks of age, the growth curve between 19 and 30 weeks of age of wild-type mice fed a low-fat diet was significantly larger than that of *CAG/orexin*-transgenic mice ($p = 0.0016$). Likewise, *OX1R*$^{-/-}$ male mice fed a low-fat diet showed larger body weight growth between 17 and 30 weeks of age than *OX1R*$^{-/-}$; *CAG/orexin* mice despite no significant difference in the growth curves before 16 weeks of age ($p = 0.036$). *OX2R*$^{-/-}$ male mice fed a low-fat diet showed larger body weight than *OX2R*$^{-/-}$; *CAG/orexin* mice through the whole observation period ($p = 0.005$); however, the fat mass and serum leptin of *OX2R*$^{-/-}$; *CAG/orexin* male mice were similar to those of *OX2R*$^{-/-}$ male mice (Figure 2).

CAG/Orexin Transgene Reduces Fat Mass and Leptin

Consistent with body weight data, at 28 weeks of age, *CAG/orexin* male mice showed a significant reduction of fat mass on a low-fat diet as compared with wild-type male mice (Figure 2A). The fat mass of *CAG/orexin* male mice and *OX1R*$^{-/-}$; *CAG/orexin* mice fed a high-fat diet was significantly less than those of wild-type mice and *OX1R*$^{-/-}$ mice, respectively, for both sexes (Figures 2A and 2B). There was no significant difference in fat mass between *OX2R*$^{-/-}$ mice and *OX2R*$^{-/-}$; *CAG/orexin* mice on both a low-fat and a high-fat diet for either sex. *OX2R*$^{-/-}$ male mice exhibited a significant tendency toward increased fat mass under high-fat conditions, and *OX2R*$^{-/-}$ female mice exhibited a mild but significant tendency toward increased fat mass under even low-fat conditions compared to wild-type mice, which is consistent with previously described adiposity of narcoleptic mice (Hara et al., 2001) and a physiological role of OX2R signaling in suppressing adiposity.

Next, we measured serum leptin, which typically correlates with fat mass. Concordance between fat mass and leptin levels was confirmed in each genotype. Specifically, the leptin levels of *CAG/orexin* mice and of *OX1R*$^{-/-}$; *CAG/orexin* mice were significantly lower than those of wild-type mice and of *OX1R*$^{-/-}$ mice fed a high-fat diet, respectively, whereas there was no significant difference in serum leptin levels between *OX2R*$^{-/-}$; *CAG/orexin* mice and *OX2R*$^{-/-}$ mice on both low-fat and high-fat diets for both sexes (Figures 2C and 2D). Compared to differences observed in fat mass, the *CAG/orexin* transgene was associated

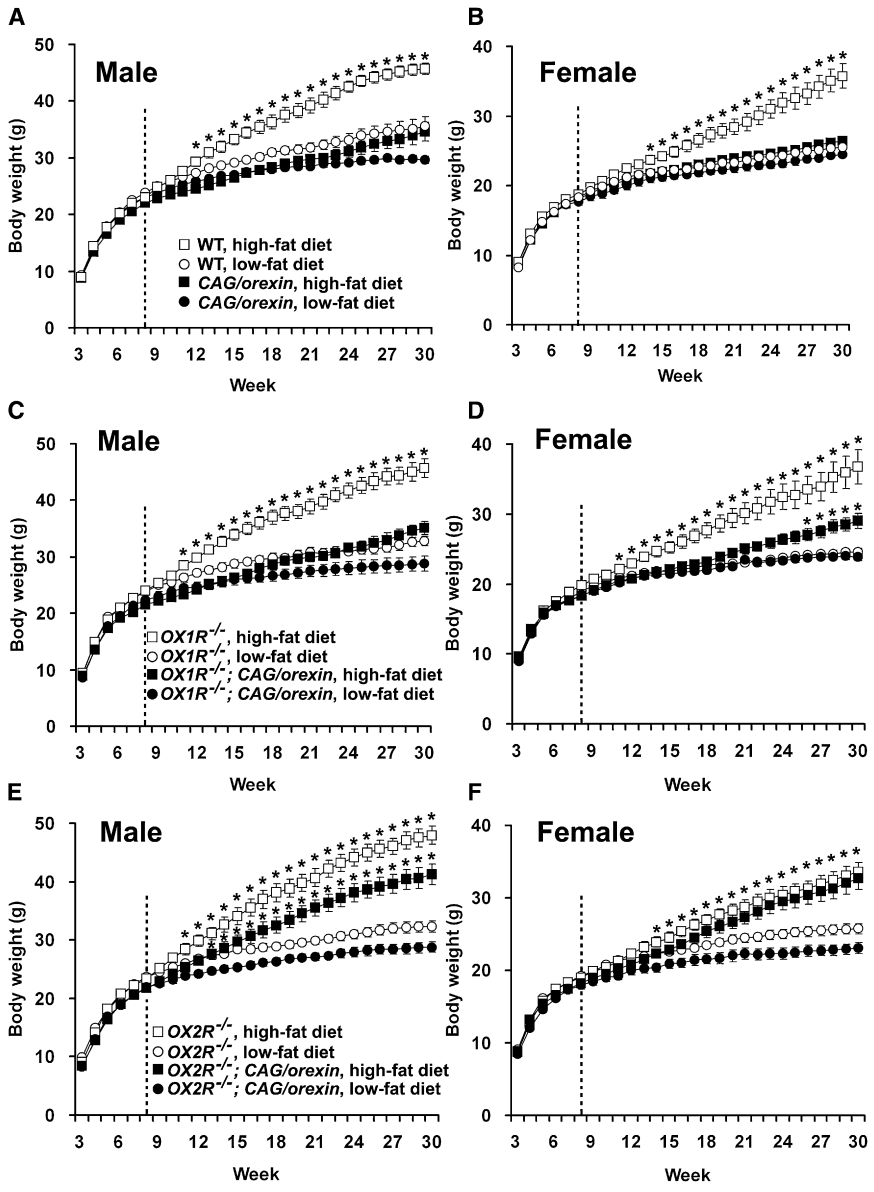


Figure 1. Growth Curves of Genetically Modified Mice Fed a Low- or High-Fat Diet

(A–F) Body weights of mice measured weekly from the age of 3 weeks to 30 weeks. A high-fat diet started at the age of 8 weeks (dotted line). The numbers of mice are 10–14 mice per group. * indicates significant difference between different diet condition for each genotypic group according to post hoc analysis at each time point. Significant differences in (F) for $OX2R^{-/-}$ mice and $OX2R^{-/-}$; *CAG/orexin* are indicated by one asterisk. Data are expressed as means ± SEM. Body weight curves of wild-type male (A) and female (B) mice on a high-fat diet were significantly higher than those of wild-type mice on a low-fat diet ($p < 0.0005$, both sexes). *CAG/orexin* mice did not differ significantly under low-fat and high-fat dietary conditions ($p = 0.51$ male; $p = 0.13$ female). Body weight curves of $OX1R^{-/-}$ male (C) and female (D) mice on a high-fat diet were significantly higher than those of $OX1R^{-/-}$ mice on a low-fat diet ($p < 0.0001$ male; $p < 0.01$ female). $OX1R^{-/-}$; *CAG/orexin* male mice did not differ significantly between low-fat and high-fat dietary conditions ($p = 0.21$). The body weights of $OX1R^{-/-}$; *CAG/orexin* female mice on a high-fat diet were significantly less than those of $OX1R^{-/-}$ mice on a high-fat diet ($p < 0.01$), in spite of no body weight difference between $OX1R^{-/-}$; *CAG/orexin* female mice and $OX1R^{-/-}$ female mice on a low-fat diet ($p = 0.50$). The body weight growths of $OX2R^{-/-}$ male (E) and female (F) mice on a high-fat diet were significantly higher than those of $OX2R^{-/-}$ mice on a low-fat diet ($p < 0.0001$ male; $p < 0.005$ female). Likewise, $OX2R^{-/-}$; *CAG/orexin* male (E) and female (F) mice showed significant weight gain on a high-fat diet compared to those on a low-fat diet ($p < 0.0005$ male; $p < 0.001$ female).

with small but significant reductions in lean mass of male mice having functional orexin receptors and those deficient in OX1R under high-fat conditions (Figure 2E). The *CAG/orexin* transgene was similarly associated with a significant mild reduction in lean mass of female mice having functional receptors under high-fat conditions, but a significant mild reduction of lean mass by the transgene under OX2R-deficient low-fat conditions was also observed (Figure 2F).

Increased Energy Expenditure of *CAG/Orexin* Mice

To explore the underlying cause of differential resistance to diet-induced obesity in mice overexpressing orexin, we housed mice from each genotypic group in metabolic cages in order to measure oxygen consumption, carbon dioxide production, and locomotor activity. The effective mass-corrected energy expenditures of *CAG/orexin* male mice and $OX1R^{-/-}$; *CAG/orexin* mice on a high-fat diet were consistently elevated over those of wild-type mice and $OX1R^{-/-}$ mice, respectively (Figures 3A, 3C, and 3G), while the energy expenditures of $OX2R^{-/-}$; *CAG/orexin* mice resembled those of $OX2R^{-/-}$ mice (Figures 3E and 3G). In contrast, we observed no consistent differences in respiratory quotient (RQ; an indirect indicator of lipid versus carbohydrate utilization) among different genotypic groups on a high-fat diet (Figures 3B, 3D, 3F, and 3H). The *CAG/orexin* transgene induced no differences in energy expenditure or RQ among any genotypic groups on a low-fat diet, regardless of the presence or absence of orexin receptors (Figure S4). Low-fat-fed $OX1R^{-/-}$ mice showed reduced energy expenditure compared to wild-type controls (Figure S4G). Importantly, *CAG/orexin* transgenic mice did not exhibit hyperactivity, regardless of diet or receptor status (Figure S5), although $OX2R^{-/-}$ mice fed a low-fat diet showed some reduced locomotion compared to wild-type mice (Figure S5G), which is consistent with previous data from narcoleptic mice (Hara et al., 2001, 2005). Basal core body temperature in *CAG/orexin* mice on a high-fat diet tended to be higher than in wild-type controls, but this difference did not reach significance (wild-type low-fat diet: 36.6°C ± 0.1°C; *CAG/orexin* low-fat

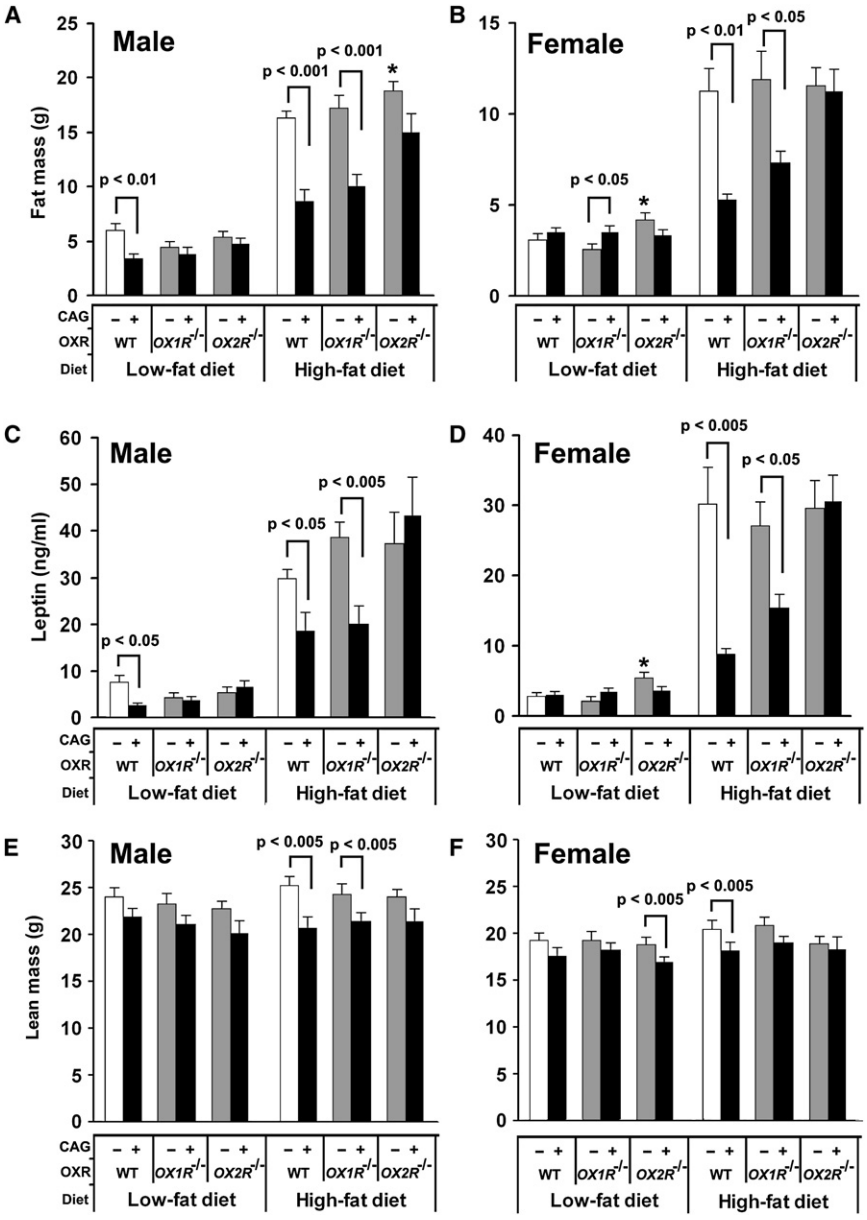


Figure 2. Fat Mass, Serum Leptin Levels, and Lean Mass of Orexin Signaling-Modified Mice

(A and B) The fat masses of male (A) and female (B) mice at 28 weeks of age on different diets.

(C and D) The serum leptin of male (C) and female (D) mice at 30 weeks of age on different diets.

(E and F) The lean masses of male (E) and female (F) mice at 28 weeks of age on different diets. * indicates significant ($p < 0.05$) increase compared to wild-type mice under the same food condition. The numbers of mice are 8–14 mice per group. Data are expressed as means ± SEM.

diet: 36.7°C ± 0.1°C; wild-type high-fat diet: 36.8°C ± 0.1°C; *CAG/orexin* high-fat diet: 37.0°C ± 0.1°C; n = 5–6).

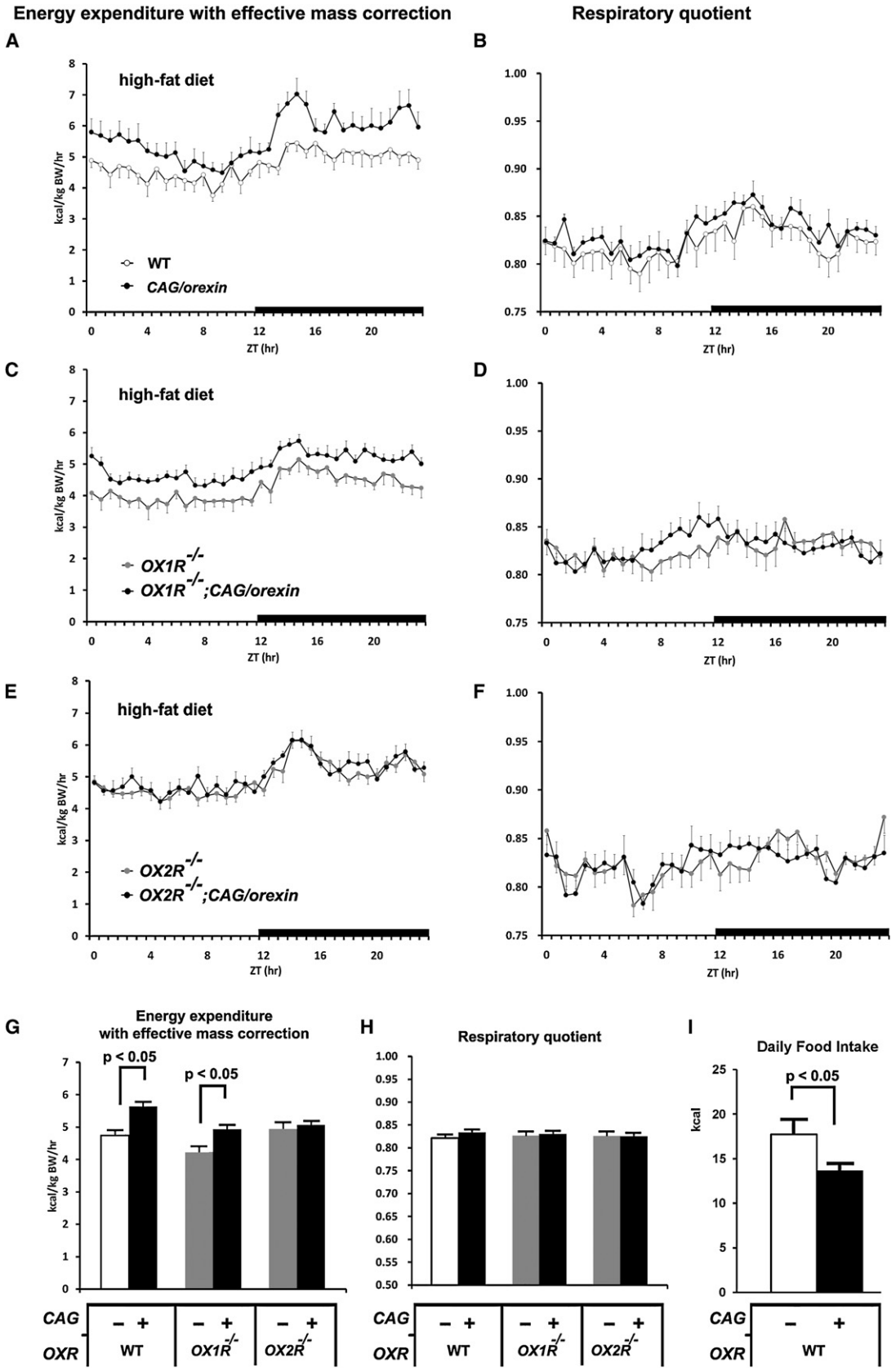
Both total high-fat diet intake for 14 days (Figure 3I) and body weight-adjusted daily food intake (data not shown) were significantly reduced in *CAG/orexin* mice compared to wild-type controls. Critically, this did not result from abnormal taste preferences: compared to wild-type mice, *CAG/orexin* and wild-type mice similarly exhibited greater preferences for high-fat over low-fat chow and for 10% sucrose over 1% sucrose solutions (Figure S6).

Glucose Metabolism of *CAG/Orexin* Mice

To examine the effect of orexin overexpression on glucose metabolism, we first measured blood glucose and serum insulin of fed mice at 30 weeks of age. When maintained on a low-fat diet, we observed no significant difference in fed glucose level among genotypes (Figure 4A). On a high-fat diet, however, wild-type mice exhibited hyperglycemia that is attenuated in *CAG/orexin*, *OX1R*$^{-/-}$, and *OX1R*$^{-/-}$; *CAG/orexin* mice, but not *OX2R*$^{-/-}$ or *OX2R*$^{-/-}$; *CAG/orexin* mice. Thus, the protective effect depends upon functional OX2R, but can be mediated by endogenous orexin levels even without orexin overexpression. Notably, these data also show that OX1R deficiency alone can prevent high-fat diet-induced hyperglycemia (see below).

Increased serum insulin levels with obesity or aging indicate mounting insulin resistance and sensitively predict deteriorating glucose control in human metabolic syndrome. When compared to wild-type mice, the *CAG/orexin* transgene reduced serum insulin levels on a low-fat diet and conferred protection from hyperinsulinemia on high-fat diet (Figure 4B). Notably, a similar protective effect occurred in *OX1R*$^{-/-}$ mice (Figure 4B), despite relative obesity under these conditions (Figures 1C, 1D, 2C, and 2D), suggesting that endogenous orexin-OX1R signaling can play a specific permissive role in development of hyperinsulinemia. However, the *CAG/orexin* transgene conferred protection from hyperinsulinemia upon all three genetic backgrounds on a high-fat diet, suggesting that both OX1R and OX2R mediate protective effects of orexin overexpression on insulin sensitivity.

We next examined the effects of orexin overexpression upon fasting glucose and glucose tolerance after glucose administration in mice. On a low-fat diet, orexin overexpression did not significantly affect glucose homeostasis ($p = 0.47$, Figure S7). On a high-fat diet, however, *CAG/orexin* mice exhibited significantly reduced basal fasting glucose levels as well as improved glucose tolerance at all time points tested, relative to wild-type controls (Figure 4C). Despite absence of basal differences in fasting serum glucose between *OX1R*$^{-/-}$ and *OX1R*$^{-/-}$; *CAG/orexin* mice, the *CAG/orexin* transgene conferred mild but significant improvements in glucose tolerance onto the *OX1R*$^{-/-}$


Energy expenditure with effective mass correction
Respiratory quotient
A
B
high-fat diet
kcal/kg BW/hr
ZT (hr)
WT
CAG/orexin
C
D
OX1R-/-
OX1R-/-;CAG/orexin
E
F
OX2R-/-
OX2R-/-;CAG/orexin
G
Energy expenditure with effective mass correction
p < 0.05
p < 0.05
H
Respiratory quotient
I
Daily Food Intake
kcal
CAG
OXR
WT
OX1R-/-
OX2R-/-

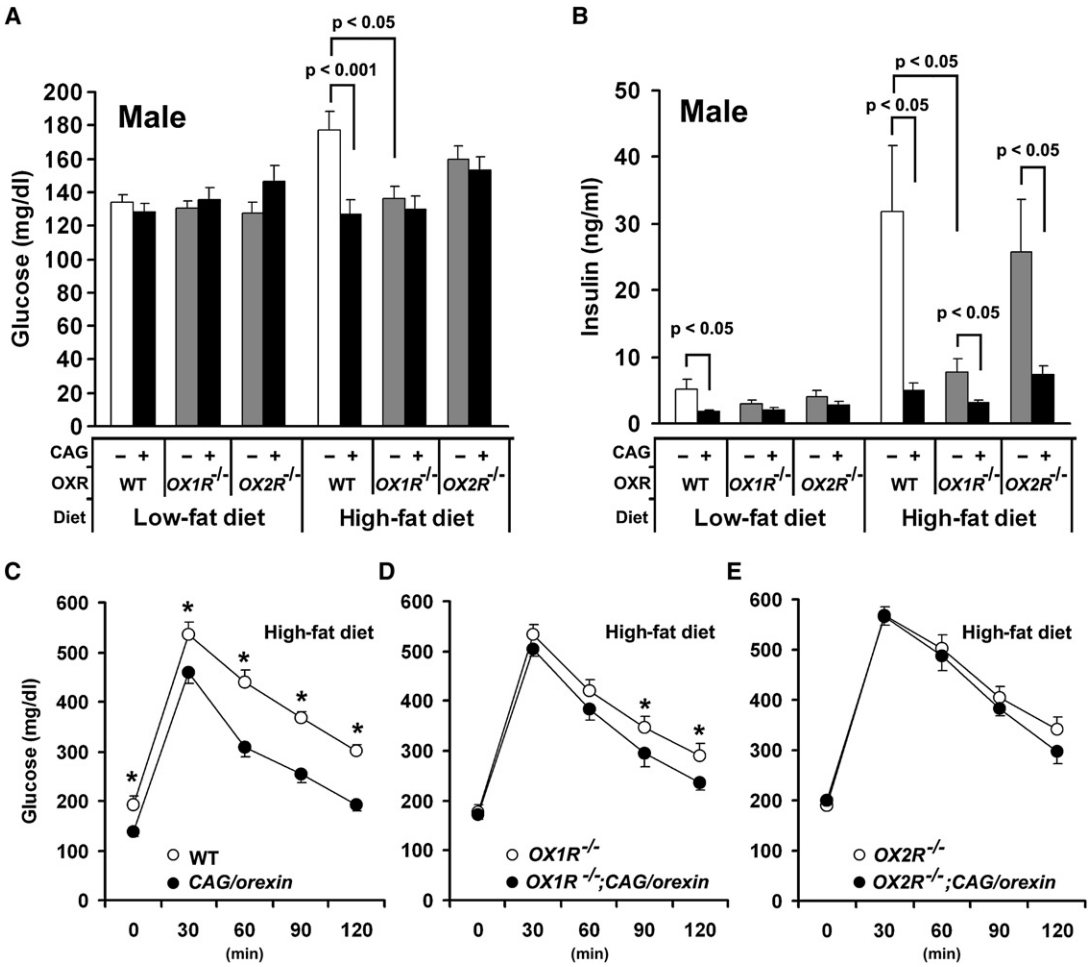


Figure 4. Glucose Metabolism of Orexin Signaling-Modified Mice on Different Fat Diets

(A) Blood glucose levels of orexin-related gene mutant mice on different fat diets.

(B) Serum insulin levels of orexin-related gene mutant mice on different fat diets.

(C) Glucose tolerance test showed that the blood glucose levels of *CAG/orexin* mice were significantly lower than those of wild-type littermate mice on a high-fat diet after the administration of glucose.

(D) Glucose tolerance test showed that the blood glucose levels of $OX1R^{-/-}$; *CAG/orexin* mice were significantly lower than those of $OX1R^{-/-}$ mice on a high-fat diet after the administration of glucose. Data are expressed as means ± SEM.

(E) Glucose tolerance test showed that there is no significant difference in the blood glucose levels between $OX2R^{-/-}$; *CAG/orexin* mice and $OX2R^{-/-}$ mice on a high-fat diet after the administration of glucose ($p = 0.33$). The numbers of mice are 8–14 mice (A and B) and 6–10 mice (C–E) per group. Data are expressed as means ± SEM (*$p < 0.05$).

background at later time points (Figure 4D). Improved glucose tolerance in the setting of reduced insulin levels indicates that the transgene confers improved insulin sensitivity. By contrast, we observed no significant differences in fasting glucose or glucose tolerance between $OX2R^{-/-}$ mice and $OX2R^{-/-}$; *CAG/orexin* mice (Figure 4E). Thus, while OX1R may also influence circulating insulin levels, orexin overexpression improves insulin sensitivity by a predominantly OX2R-dependent mechanism.

Effects of *CAG/Orexin* Transgene on Peripheral Tissues

Ectopic orexin production in thyroid tissue raises the possibility that abnormal activity of the thyroid axis contributes to leanness

Figure 3. The Metabolic Parameters of Orexin Signaling-Modified Mice on a High-Fat Diet

(A and B) The energy expenditure with effective mass correction (A) and respiratory quotient (B) sampled every 40 min over 24 hr of *CAG/orexin* mice and wild-type mice at 16–20 weeks of age.

(C and D) The energy expenditure with effective mass correction (C) and respiratory quotient (D) over 24 hr of $OX1R^{-/-}$; *CAG/orexin* mice and $OX1R^{-/-}$ mice.

(E and F) The energy expenditure with effective mass correction (E) and respiratory quotient (F) over 24 hr of $OX2R^{-/-}$; *CAG/orexin* mice and $OX2R^{-/-}$ mice.

(G and H) The averaged energy expenditure with effective mass correction (G) and respiratory quotient (H).

(I) Averaged daily high-fat diet intake of *CAG/orexin* mice and wild-type mice for 14 days. The numbers of mice are 6–9 mice per group. Data are expressed as means ± SEM.

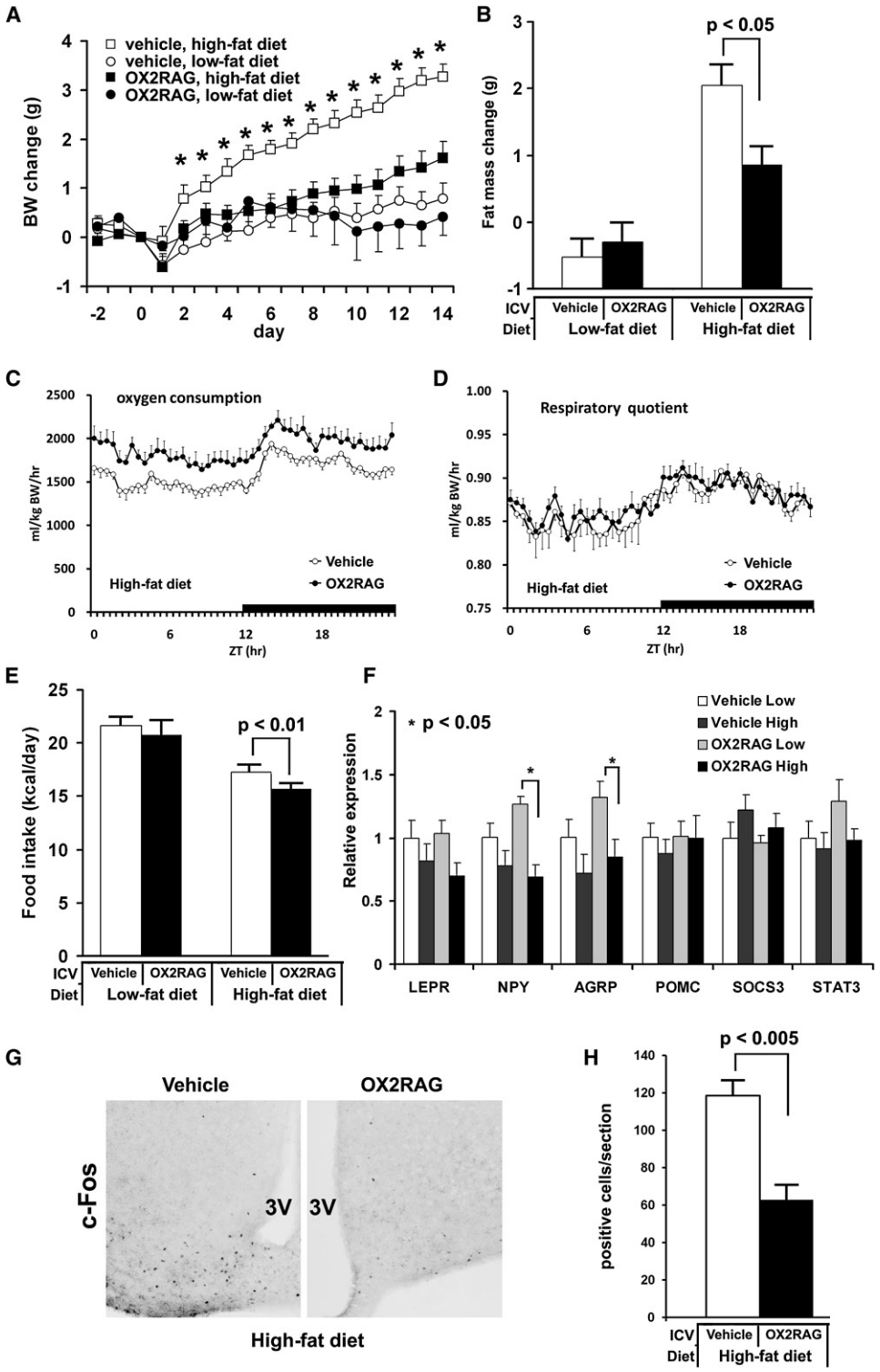


Figure 5. Effect of OX2R Selective Agonist on Diet-Induced Obesity

(A) The daily body weight changes of chronically ICV-injected mice. ICV and high-fat diet begin at day 0. The body weight growths of the OX2R selective agonist-injected mice (0.5 nmol/day) are significantly lower than those of vehicle-injected mice on a high-fat diet ($p < 0.0005$), whereas there is no significant difference in the body weight growth between them on a low-fat diet ($p = 0.45$).

(B) The fat mass change after the infusion of OX2R-selective agonist or vehicle for 14 days under a low- or high-fat diet.

(C) The oxygen consumption with effective mass correction of OX2R agonist-infused mice on a high-fat diet was higher than vehicle-infused mice ($p < 0.0005$, repeated ANOVA). Data were sampled every 30 min.

in *CAG/orexin* mice. We measured serum thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) on low- and high-fat diets. High-fat diet increased serum T3 and T4 levels of *CAG/orexin* mice to an extent similar to wild-type mice despite significant differences in adiposity between the groups (Figure S8). Serum TSH levels of *CAG/orexin* mice on a high-fat diet were significantly elevated over those on a low-fat diet, while a high-fat diet did not significantly affect serum TSH levels of wild-type mice. Importantly, the levels of serum TSH, T3, and T4 of *CAG/orexin* mice were similar to those of wild-type mice when maintained on a low-fat diet.

To determine whether increased energy expenditure of *CAG/orexin* mice was associated with increased mitochondrial uncoupling proteins, we examined mRNA levels of major uncoupling proteins in brown fat and skeletal muscle (Figure S9). High-fat diet resulted in comparable increases in *UCP1* mRNA in brown fat, but not skeletal muscle, in both genotypes. In contrast, *UCP2* and *UCP3* mRNA levels did not differ significantly by genotype or dietary condition, consistent with previous reports (Surwit et al., 1998).

Despite detection of ectopic orexin peptide in adrenal gland, *CAG/orexin* mice and wild-type mice had similar total daily urinary levels of epinephrine and norepinephrine and similar serum corticosterone levels (Figure S10). In addition, *CAG/orexin* transgene did not affect systolic blood pressure on either a low-fat or high-fat diet (Figure S10).

OX2R Agonist Prevents Diet-Induced Obesity

Our genetic studies implicate the OX2R pathway as mediator of the effects of orexin overexpression upon energy homeostasis. To further test the hypothesis that central enhancement of orexin-OX2R signaling confers resistance to diet-induced obesity, an OX2R selective agonist [Ala11, D-Leu15] Orexin-B (Asahi et al., 2003) was continuously infused in the lateral ventricles of wild-type mice for 14 days. The administration of the OX2R selective agonist suppressed weight gain on a high-fat diet without altering weight homeostasis on a low-fat diet (Figure 5A). Importantly, the OX2R selective agonist had no obvious effect upon OX2R-deficient mice on a high-fat diet ($n = 4$, weight gain 3.33 ± 0.61 g, $p = 0.67$), verifying the specificity of the agonist in vivo. Following 14 days, the agonist-infused wild-type mice gained significantly less fat mass than did the vehicle-injected mice on a high-fat diet, and no effect was observed on a low-fat diet (Figure 5B). When centrally infused mice fed a high-fat diet were monitored in metabolic chambers, OX2R agonist infusions resulted in consistently greater energy expenditures (Figure 5C), but not RQs (Figure 5D) or locomotor activity (data not shown), over vehicle-infused controls.

As sleep/wake disturbances could affect food intake and energy expenditure, we recorded EEG/EMG signals during central OX2R agonist or vehicle infusions. Mice receiving OX2R agonist exhibited total wake or sleep times during both light and dark phases that closely resembled vehicle controls, irrespective of dietary condition (Figure S11). As predicted from previous studies (Willie et al., 2003), OX2R agonism continued to promote consolidation of behavioral states, as demonstrated by increased wake and NREM episode durations in mice maintained on a low-fat diet. As this consolidation was not evident under high-fat-fed conditions, sleep/wake change cannot be the primary cause in metabolic effects of enhanced orexin signaling observed predominantly under high-fat conditions.

We observed an expected homeostatic reduction of food intake in mice maintained on a high-fat diet compared to a low-fat diet (West et al., 1992), and administration of the agonist significantly enhanced this effect by further suppressing food intake selectively in mice fed a high-fat diet (Figure 5E). After 14 days of OX2R agonist administration, we observed reduced hypothalamic mRNA expression of orexigenic factors *NPY* and *AGRP* on a high-fat diet compared to those on a low-fat diet (Figure 5F). Indeed, the number of c-Fos-positive cells in ARH region was significantly reduced in OX2R agonist-administered mice on a high-fat diet (Figures 5G and 5H). The reduction of c-Fos-positive cell number was particularly notable in the ventromedial aspect of ARH (Figure 5G), in which orexigenic NPY/AGRP neurons are located (Horvath, 2005), consistent with the observed reduction in food intake and in *NPY/AGRP* mRNAs we observed under this condition.

Leptin Mediates Antiobesity Effects of Orexin

Leptin negatively regulates body weight, suppresses food intake, and increases energy expenditure by inhibiting NPY/AGRP neurons and activating POMC neurons of ARH. Diet-induced obesity is associated with leptin resistance resulting from signal transduction abnormalities in ARH (Myers et al., 2008). OX2R is highly expressed in ARH, and the effects of circulating leptin upon ARH resemble some effects of increased orexin-OX2R signaling that we observed. We hypothesized, therefore, that leptin signaling mediates some of the metabolic effects of orexin. To examine the consequences of orexin signaling enhancement on mice in the absence of leptin activity, we crossed *CAG/orexin*-transgenic and leptin-deficient *ob/ob* lines. Remarkably, the *CAG/orexin* transgene had no impact upon weight gain or fat mass of leptin-deficient *ob/ob* mice (Figures 6A and 6B), suggesting that, indeed, the antiobesity effect of *CAG/orexin* depends upon leptin activity. We then centrally administered OX2R agonist to *ob/ob* mice and similarly found no significant effect upon weight gain under low- or high-fat dietary conditions (Figure 6C). We also observed no effect of OX2R agonist compared to vehicle administration upon core body temperature of *ob/ob* mice (data not shown).

We directly examined whether orexin overexpression alters sensitivity to leptin. Leptin was continuously administered in

(D) The respiratory quotient of OX2R agonist-infused mice on a high-fat diet was similar to that of vehicle-infused mice.
(E) The average daily food intake of mice injected with the OX2R selective agonist or vehicle on different fat diets during 14 days.
(F) Hypothalamic gene expressions at the end of OX2R selective agonist administration are determined using q-PCR. Gene expressions are normalized by GAPDH.
(G) Immunostaining for c-Fos in ARH region of mouse on a high-fat diet during central administration of OX2R agonist or vehicle; 3V, third ventricle.
(H) The number of c-Fos-positive cells in ARH region. The numbers of mice per group are 7–14 mice for (A), (B), and (C); 6–7 mice for (D), (G), and (H); and 5–6 mice for (E) and (F). Data are expressed as means ± SEM.

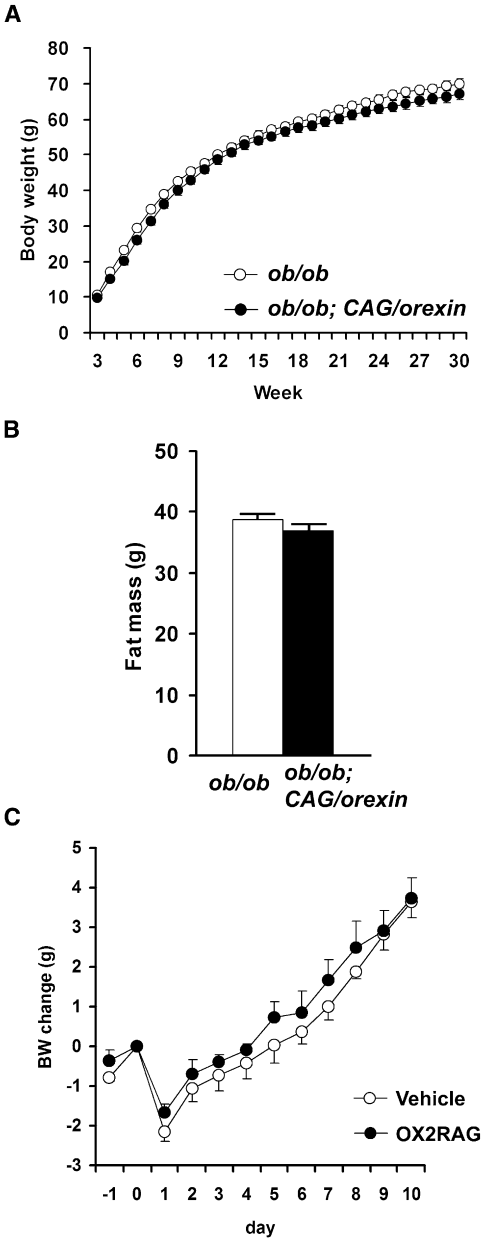


Figure 6. No Effect of Orexin Overexpression on the Weight Gain of *ob/ob* Mouse

(A) The body weight growth of *ob/ob*; *CAG/orexin* male mice was similar to that of *ob/ob* male mice ($p = 0.76$).

(B) Fat mass of 28-week-old *ob/ob* male mice with or without *CAG/orexin* transgene. There is no significant difference in fat mass for both male and female. There were 13–20 mice per group.

(C) The body weight growth of OX2R agonist-infused *ob/ob* mice was similar to that of vehicle-injected *ob/ob* mice maintained on a low-fat and a high-fat diet. Data are expressed as means ± SEM.

the lateral ventricles of *CAG/orexin* and wild-type littermate pairs. Mice (3–4 months old) were maintained on a low-fat diet in order to initially match as to body weight (WT 29.7 ± 3.6 ng/μl and *CAG/orexin* 27.0 ± 3.1 ng/μl). Both wild-type mice and *CAG/orexin* mice lost weight during the administration of leptin, but *CAG/orexin* mice showed significantly enhanced weight loss and anorexia compared to wild-type mice on a low-fat diet (Figures 7A and 7B), indicating that increased orexin signaling is associated with a more leptin-sensitive state.

Compared to control wild-type mice, 14 days of central leptin administration resulted in basal hypothalamic expression levels of *NPY* and *AGRP* and an expected induction of *POMC* mRNA (Figure 7C). In contrast, under basal conditions, *CAG/orexin* transgenic mice showed increased expression of *NPY* and *AGRP*, but not *POMC* mRNA. While we detected no significant changes in the expression of LEPR, SOCS3, or STAT3 gene products, the overall profile of altered hypothalamic gene expression we detected is consistent with the physiological state of anorexia and weight loss observed in mice undergoing leptin administration.

DISCUSSION

When challenged with a high-fat diet, *CAG/orexin* mice maintain elevated energy expenditure, decreased food intake, and resistance to diet-induced obesity, hyperleptinemia, and hyperinsulinemia, although these mice show normal adiposity and energy homeostasis under a low-fat diet. Molecular genetic dissection of the metabolic phenotype utilizing *CAG/orexin*; *OX1R*$^{-/-}$ and *CAG/orexin*; *OX2R*$^{-/-}$ mice indicated that OX2R predominantly mediates these antiadipogenic effects and improves insulin sensitivity. Central infusion of an OX2R agonist confirms the role of central orexin-OX2R signaling in protection from high-fat diet-induced obesity. Furthermore, the antiadipogenic effects of genetic or pharmacologic enhancement of orexin signaling require leptin, and *CAG/orexin* mice exhibit increased sensitivity to exogenous leptin infusion.

Technical Considerations Regarding *CAG/Orexin* Transgene

The *CAG* promoter is a universal, constitutively active promoter, yet ectopic orexin production was restricted to a limited number of tissues, likely due to the necessity of lineage-specific enzymatic machinery required for neuropeptide production. Likewise, immunohistochemical localization of orexin-A peptide does not demonstrate homogenous presence of the antigen throughout brain parenchyma, but restricted presence at specific brain regions. In *CAG/orexin* mice, the appearance and number of strongly orexin-A-positive neurons in LHA (endogenous orexin cells) and the density of orexin-A-positive fibers in brain regions to which endogenous orexin neurons normally project resemble wild-type mice (Figure S2). Additionally, we observe a diffuse background ectopic orexin-A immunoreactivity, especially in the medial basal hypothalamus, including the ARH (Figures S1 and S2). Ectopically expressed orexin confers physiologic signaling, which is demonstrated by our previous result that the *CAG/orexin* transgene rescues the narcolepsy-cataplexy phenotype (Mieda et al., 2004). Moreover, dependency of the *CAG/orexin* phenotype upon the intact OX2R gene demonstrates that the metabolic phenotype of *CAG/orexin* mice is not an artifact, but the physiological effect of increased orexin-OX2R signaling.

Although we cannot rule out a contribution of ectopic orexin production in the thyroid, adrenal medulla, and pancreatic islets to the phenotype of *CAG/orexin* mice, we found no indication of

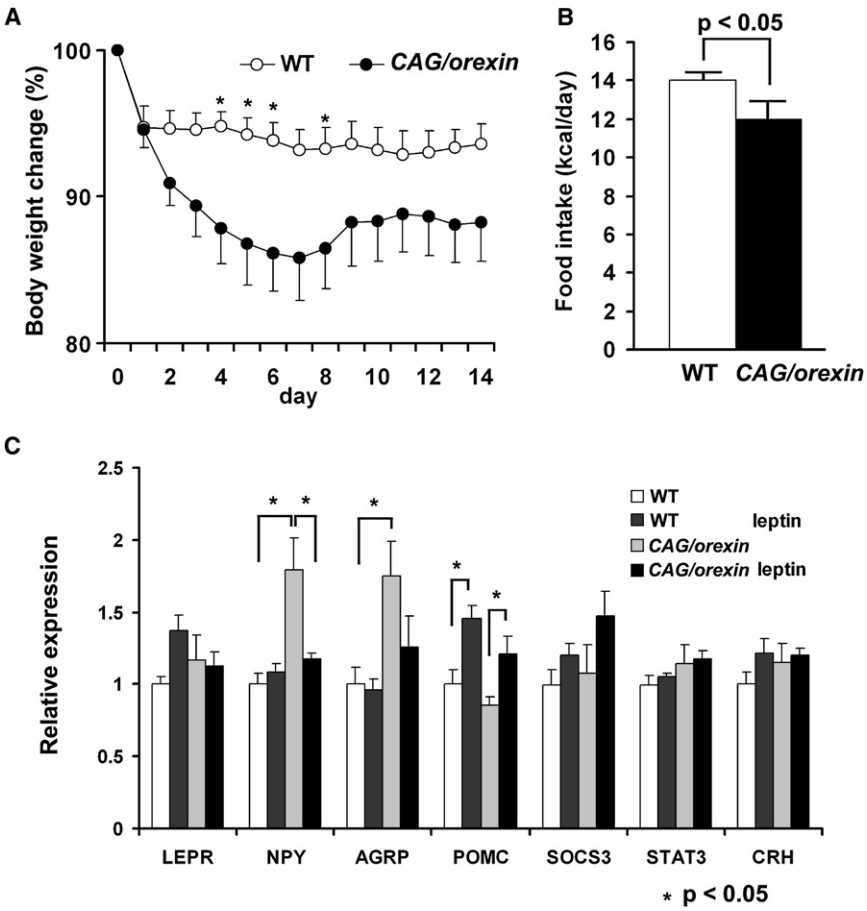


Figure 7. Increased Sensitivity of Orexin Overexpression Mouse to Leptin

(A) The body weight changes during chronic ICV injection of leptin (2 μg/day) for 14 days. *CAG/orexin* mice on a low-fat diet show larger weight loss compared with wild-type mice ($p < 0.05$).

(B) Daily food intake of *CAG/orexin* mice during chronic injection of leptin is smaller than that of wild-type mice.

(C) Hypothalamic gene expressions at the end of leptin administration. There were 6–8 mice per group. Data are expressed as means ± SEM (*$p < 0.05$).

primary peripheral endocrine disturbance in *CAG/orexin* mice. *CAG/orexin* mice have normal levels of serum TSH, T3, T4, corticosteroid, and urinary catecholamines, and we observed improved glucose metabolism consistent with increased insulin sensitivity and reduced leptin levels that correspond in the expected manner to reduced adiposity. Furthermore, replication of antiadipogenic effects with central OX2R agonist administration suggests that the metabolic effects of enhanced orexin signaling originate centrally.

Orexin Signaling Promotes Negative Energy Balance

Energy balance is a function of caloric intake and energy expenditure, and our data indicate that both orexin overexpression and OX2R agonist infusion increase energy expenditure and further suppress consumption of a high-fat diet, providing the mechanistic rationale for observed resistance to adiposity. While this result seems at odds with the well-documented acute pharmacologic orexigenic activity of orexin (Sakurai et al., 1998; Willie et al., 2001), chronic central administration of orexin-A does not support increased food consumption or anabolism in rats (Yamanaka et al., 1999). This suggests that the acute appetite-promoting effects of orexin peptides may be temporary, or progressively overwhelmed by counterregulatory mechanisms that oppose weight gain.

Low-fat-fed mice carrying the *CAG/orexin* transgene or treated with OX2R agonist for 2 weeks demonstrated elevated expression of the orexigenic genes *NPY* and *AGRP.* These changes could represent a direct effect, as orexin acutely stimulates neurons of the ARH when microinjected (van den Top et al., 2004; Yamanaka et al., 2000), and orexin-stimulated food consumption depends pharmacologically upon NPY signaling (Yamanaka et al., 2000). An alternative mechanism for upregulated *NPY* and *AGRP* transcription could be a compensatory response to relative negative energy balance, since we noted significant differences from controls only when mice were maintained on calorie-poor rather than calorie-dense chow.

Despite differences in orexigenic effects across different experimental paradigms, consistent and unifying results from pharmacologic and genetic studies indicate that orexin gain of function promotes energy expenditure while loss of function promotes energy conservation. Just as the orexin system is believed to orchestrate disparate circuits of the ascending arousal system to maintain a consolidated state of arousal, it may also normally serve to consolidate the activity in parallel reward and metabolic networks that control behavioral and homeostatic responses to support energy expenditure. The exact peripheral (downstream) mechanisms for the orexin-mediated increases of energy consumption remain unclear. Although we did not detect significant increase in urinary catecholamines or basal blood pressure, the data do not exclude the possibility of a subtly increased sympathetic tone in certain peripheral tissues. Indeed, we speculate that the sympathetic pathways are one of likely downstream mechanisms for the increased metabolic rate under enhanced orexin signaling.

Interactions of Orexin and Leptin Signals

Antiadipogenic effects of orexin-OX2R signaling require the presence of leptin, and orexin-overexpressing mice showed increased sensitivity to catabolic-anorectic effects of exogenous leptin. These findings suggest that leptin mediates the suppressive effect of enhanced OX2R signaling on diet-induced obesity. Leptin-responsive neurons are found in ARH, VMH, DMH, LHA, and tuberomamillary nucleus of the hypothalamus (Elmquist,

2000). These nuclei receive orexin innervations, express high levels of OX2R, and exhibit ectopic orexin immunostaining in *CAG/orexin*-transgenic mice. Among these, ARH is a particularly critical nexus for body weight regulation that monitors peripheral energy storages and enteral feeding status through integration of circulating leptin and insulin, metabolites, and vagal relays. Through outputs to other hypothalamic and brainstem sites, ARH modulates the thresholds, triggering drives to eat and expend energy, and it influences insulin secretion and sensitivity (Horvath, 2005; Coppari et al., 2005; Myers et al., 2008). Moreover, ARH harbors cellular abnormalities underlying acquired leptin resistance (Kievit et al., 2006), and reduced leptin sensitivity in ARH has been causally linked with diet-induced obesity (Enriori et al., 2007). While ARH neurons project to orexin neurons of the LHA, ARH receives dense reciprocal orexin fiber innervation and expresses mainly OX2R receptor (Cluderay et al., 2002; Peyron et al., 1998; Marcus et al., 2001). Acute microinjections of orexin-A into ARH increase oxygen consumption and body temperature under anesthesia (Wang et al., 2003).

The mechanism by which orexin and leptin signals interact remains unclear. Neurons expressing both LEPR and OX2R may have convergent intracellular second messenger signaling, including extracellular factor-regulated kinase (ERK) and the Janus kinase JAK2/STAT3 pathways (Myers et al., 2008; Zhu et al., 2003). In ARH, leptin-responsive neurons such as those expressing NPY/AGRP are directly excited by orexin while POMC neurons are directly inhibited by orexin (Muroya et al., 2004), but inhibitory GABAergic interneurons in ARH may also be activated via postsynaptic OX2R (Burdakov et al., 2003), predicting complexity in up- or downregulation of these circuits. Fos immunostaining reveals reduced neuronal activity in a population of ARH neurons following 2 weeks of OX2R agonist administration on a high-fat diet, but the true molecular identity of these cells and the direct versus indirect nature of this effect requires investigation.

Orexin neurons could directly sense lipids through kinetics of fatty acid metabolites to alter feeding behavior and energy homeostasis. However, we observed *CAG/orexin*-transgenic mice to be also resistant to aging-associated adiposity, even when maintained on a low-fat diet, and we detected no significant differences in circulating cholesterol or fatty acids among genotypes or dietary conditions (data not shown). Therefore, abnormal kinetics of hypothalamic fatty acid metabolism alone is unlikely to explain the obesity-resistant *CAG/orexin* phenotype. Furthermore, endogenous orexin neurons are themselves unlikely to play a crucial lipid-sensing role in energy homeostasis, as *CAG/orexin* mice in which endogenous orexin neurons have been selectively eliminated remain lean (J.T.W. and T.S., unpublished data).

Potential Role of OX1R on Glucose Metabolism

The unifying scheme in the present study is that OX2R, but not OX1R, is the primary receptor that mediates the beneficial effects of orexin gain of function under a high-fat diet. The observed improvements in glucose metabolism and insulin sensitivity could largely be explained by the OX2R-mediated reduction of body adiposity. However, there is a notable exception: we observed that OX1R deficiency alone, without orexin overexpression, can improve glycemia and insulin sensitivity on a high-fat diet (Figures 4A and 4B), despite the fact that *OX1R*$^{-/-}$ and wild-type mice are similarly obese under a high-fat diet (Figures 1C and 2C). This suggests that endogenous levels of orexin acting on OX1R may, in part, mediate the deleterious effects of high-fat diet on glucose metabolism. Indeed, OX1R is expressed in the solitary tract nucleus and dorsal motor nucleus of the vagus (Marcus et al., 2001), which participates in the regulation of hepatic glucose production (Pocai et al., 2005). Although OX1R is also detected in beta cells of pancreatic islets, a role for orexin, if any, in the pancreatic islet is controversial (Heinonen et al., 2008). At any rate, under orexin overexpression, the OX2R-mediated effects prevail, and the presence or absence of OX1R does not affect (the improvement of) glycemia or insulinemia. The specific role of OX1R on glucose regulation merits further investigation.

Therapeutic Implications

The robust innervation by the orexin system of the whole brain and the multiple phenotypic aspects of orexin-deficient animals such as cataplexy, attenuated morphine dependence, and diminished stress response has led to conceptualization of the orexin system as a hypothalamic output pathway controlling arousal, motivational behavior, and autonomic responses (Chemelli et al., 1999; Sakurai, 2007). Our results demonstrate that orexin signaling also has the capacity to primarily promote energy expenditure via leptin sensitization. Augmentation of OX2R signaling or its downstream targets beneficially alters hypothalamic setpoints controlling metabolic rate, food intake, and leptin and insulin sensitivity. Similar interventions in humans might prevent or reverse the effects of consumption of calorie-dense food that promote or maintain pathological adiposity and metabolic syndrome. From a therapeutic standpoint, it is important to note that orexin gain of function did not overtly alter the basal blood pressure, or the thyroid, glucocorticoid, and catecholiamine statuses in our models.

While continuous orexin gain of function did not induce locomotor hyperactivity or perturb overall amounts of sleep and wakefulness in *CAG/orexin* mice or OX2R agonist-infused mice, further sleep/wake characterization of these models is warranted. The metabolic syndrome is a disorder not only of obesity and insulin insensitivity, but possibly also inactivity, sleep/wake disturbances, and comorbid depression (Fabricatore and Wadden, 2006). Daytime administration of an OX2R agonist to such individuals could have multiple beneficial effects by maintaining elevated metabolic rate while also promoting daytime wakefulness and consolidating sleep/wake states. The orexin system has emerged as a key target for therapeutic intervention in disorders associated with hypothalamic dysfunction, including not only narcolepsy and hypersomnia, but now also the metabolic syndrome.

EXPERIMENTAL PROCEDURES

Animals

All mice were backcrossed more than ten generations to the C57BL/6J strain. In *CAG/orexin*-transgenic mice, the expression of prepro-orexin is controlled by the chimeric *CAG* promoter constructed from the chicken β-actin promoter and the cytomegalovirus immediate early gene enhancer (Mieda et al., 2004). Each genotypic group was compared by pairing of littermates as follows: wild-type with *CAG/orexin*, *OX1R*$^{-/-}$ (Kisanuki et al., 2001) with *OX1R*$^{-/-}$; *CAG/orexin*,

OX2R$^{-/-}$ (Willie et al., 2003) with *OX2R*$^{-/-}$; *CAG/orexin*, *ob/ob* with *ob/ob*; *CAG/orexin*. *Ob/ob* (Zhang et al., 1994) mice were obtained from Jackson Laboratory. Mice were provided food and water ad libitum, maintained on a 12 hr light/dark cycle at all times, and housed at two or three mice per cage under controlled temperature and humidity, unless otherwise specified. All procedures were approved by the appropriate institutional animal care and use committees and were carried out in strict accordance with NIH guidelines.

Body Weight Study

For diet-induced obesity, all mice were fed a low-fat diet (standard chow 8664 F6 Rodent Diet; Harlan Teklad) until 8 weeks of age. At 8 weeks of age, mice were assigned randomly to either a low-fat or a high-fat diet (D12451; Research Diets). A low-fat diet provided 4.1 kcal/g of energy (67% carbohydrate, 20% protein, and 13% fat). A high-fat diet provided 4.7 kcal/g of energy (35% carbohydrate, 20% protein, and 45% fat). Body weight was measured weekly until 30 weeks of age. At 28 weeks of age, mice were subjected to NMR (Minispec NMR Analyzer; Bruker) to measure fat and lean mass per the manufacturer's instructions. At 30 weeks of age, mice were euthanized to collect blood and measure blood glucose. Serum was collected from centrifuged blood and stored at −80°C until use. *Ob/ob* and *ob/ob; CAG/orexin* mice were fed a low-fat diet until 30 weeks of age.

Blood Analysis

For analysis of serum, we used Mouse Leptin and Ultra Sensitive Rat Insulin ELISA kits with Mouse Insulin Standard (Crystal Chem). Whole blood glucose levels were measured using a standard clinical glucometer (Elite; Bayer). For glucose tolerance tests, 21- to 25-week-old mice were fasted for 12 hr from ZT16 and then injected with glucose (1.5 g/kg of body weight, i.p.) at ZT4. Tail blood was collected at 0, 15, 30, 60, and 90 min after injection.

Metabolic Cage Study

Indirect calorimetry and locomotor data were simultaneously measured using the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments). For genetic studies, 16- to 20-week-old animals were individually housed in calorimeter chambers, and 3 days of data collection followed a 4 day acclimatization period. For pharmacologic studies, ICV cannulation surgery was performed at 10–12 weeks of age, and high-fat feeding initiated in an acclimatization chamber. After 4 days of acclimatization, each animal was housed in a metabolic chamber for 4.5 days. We calculated metabolic parameters based on the following equations:

$$\mathrm{RQ} = \mathrm{CO_2\ production/oxygen\ consumption}.$$

$$\text{Raw Energy Expenditure (REE)} = (3.815 + 1.232 \times \mathrm{RQ}) \times \text{oxygen consumption}.$$

$$\text{Energy Expenditure with Effective Mass Correction} = \mathrm{REE}/(\text{weight/mass unit})^{\text{effective mass factor}}.\ \text{Effective mass factor} = 0.75.$$

Chronic ICV Injection

Three- to four-month-old male C57B/6J mice were single-housed 1 week before surgery and fed a low-fat diet. Mice were anesthetized with ketamine and xylazine (100 mg/Kg and 10 mg/Kg, respectively, i.p.). A cannula (Brain Infusion Kit III; Alzet) was implanted into the right lateral ventricle (0.3 mm posterior from the bregma, 0.9 mm lateral from the midline, and 2.4 mm from the surface of skull) using standard sterile stereotactic techniques. An osmotic minipump (model 2001; Alzet) was attached to the cannula and implanted in the subcutaneous space during the same surgical session. The OX2R selective agonist ([Ala11, D-Leu15] Orexin-B; American Peptide) (Asahi et al., 2003) or vehicle was continuously injected in the lateral ventricle for 14 days (0.5 nmol/day). The agonist was diluted with vehicle (Dulbecco's PBS; Sigma) immediately before use. At the day of surgery, the implanted mice were randomly assigned to a low-fat diet or a high-fat diet. Body weight and food intake were monitored daily for 14 days, and fat mass was detected by NMR immediately after surgery and again at day 14. Twelve-week-old *ob/ob* male mice were used for chronic ICV infusion of OX2R agonist for 10 days as described above. For leptin administration experiments, weight-matched 3- to 4-month-old *CAG/orexin* and wild-type littermates were continuously injected with leptin (2 μg/day; PreproTech), as described above, while maintained on a low-fat diet. Body weight and food intake were monitored daily for 14 days.

Quantitative PCR

Hypothalamus was dissected coronally under microscope from the optic chiasm to the mamillary bodies. The thick coronal section was further trimmed bilaterally at 1 mm from the midline and dorsally at 1.5 mm from the ventral surface. This dissected tissue included ARH, VMH, DMH, PVN, anterior hypothalamic area, and a part of LHA. Total RNA was isolated using RNeasy Mini Kit and used for cDNA synthesis by random hexamer and Omniscript Reverse Transcriptase (QIAGEN). Real-time quantitative PCR reactions were performed on cDNA with ABI Prism 7000 Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's manual. *GAPDH* mRNA level was used for normalization.

Immunohistochemistry

After 2 weeks of continuous administration of OX2R agonist or PBS, mice on a high-fat diet were harvested during early dark phase under a red light. The immunohistochemistry for c-Fos was performed using free-floating method, as described previously (Chemelli et al., 1999), utilizing anti-c-Fos polyclonal antisera (Ab-5; Oncogene). Fos-positive cells in ARH region of two sections per animal were counted by an observed blinded-to-treatment group.

Data Analysis

Body weight growths and glucose tolerance tests were examined using repeated-measure analysis of variance (ANOVA) followed by Tukey's post hoc test, except where otherwise specified.

SUPPLEMENTAL DATA

Supplemental Data include 11 figures, two tables, and Supplementary References and can be found online at http://www.cell.com/cellmetabolism/supplemental/S1550-4131(08)00351-3.

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